DEIODINATION OF THYROXINE BY TWO NADP-DEPENDENT RAT LIVER MICROSOMAL DEIODINASE ACTIVITIES

Benjamin D. L. Li

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Deiodination Of Thyroxine By Two NADP-Dependent Rat Liver Microsomal Deiodinase Activities

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

By
Benjamin D.L. Li
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Abstract

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The deiodination of thyroxine (3,3',5,5'-tetraiodothyronine, T_4) by extrathyroidal tissues constitutes a critical pathway for the generation of the active metabolite, T_3 (3,3',5-triiodothyronine), as well as such species as reverse T_3 (3,3',5'-triiodothyronine) and further degradative products of the triiodothyronines. In our laboratory, solubilization of rat liver microsomes by a zwitterionic detergent, (3-((3-cholamidopropyl)-dimethylammonio)-1-propane sulfonate (CHAPS), revealed deiodinase activity detected by the generation of free ^{125}I from ^{125}I-T_4. Further purification of the CHAPS-solubilized microsomal deiodinase activity by acidification and by DEAE-Sephadex A25 column chromatography resulted in the separation of the activity into two. The first peak of activity eluted as the "breakthrough" and "wash" fractions (deiodinase A) and was stimulated by the addition of NADP and DTT. The second peak of activity eluted with 0.4 M NaCl and ovalbumin (1.0 mg/ml) as the "eluate" fractions (deiodinase B) and was stimulated by NADP in the presence of phospholipids. Using a T_3-specific radioimmunoassay, the generation of T_3 was detected in deiodinase A which was enhanced by the addition of NADP and DTT. This finding supported the results of the T_4 deiodinase assay. Much less T_3 was detectable by radioimmunoassay in deiodinase B incubated with T_4 with and without NADP and phospholipids. The generation of T_3 by both deiodinase activities A and B was less than 1% of the amount of free ^{125}I released from ^{125}I-T_4. This is consistent with the hypothesis that thyroxine is converted to T_3 and rT_3 and subsequently into further degradative products. The results presented here provide a basis for further studies on the purification of deiodinase activities by solubilization of microsomes with CHAPS.

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Introduction

The thyroid gland, derived from the Greek word, thyreos, meaning shield, is an endocrine organ whose secretion, the thyroid hormones, are involved in the regulation of cellular metabolism. The gland, as seen from the front, resembles more a butterfly than a shield. Developed as an evagination from the floor of the embryonic pharynx, the thyroid gland descends into the neck, finally resting with the upper margin of the isthmus at the level just below the cricoid cartilage. By the end of the third month of fetal development, colloid-containing follicles are visible and the release of thyroid hormones begins (16).

The functional unit of the thyroid gland is the follicle. Microscopically, the follicle appears as a spheroidal structure with a single epithelial cell layer surrounding a colloid-containing lumen. The colloid is generally a homogenous solution, principally consisting of thyroglobulin, the iodoglycoprotein from which the thyroid hormones are derived. It is thus not surprising that the distribution of follicle size is in part a determinant of the kinetics of iodine turnover in the gland to stimulants (27,53).

Synthesis of iodothyronines :

The physiologically active thyroid hormones, thyroxine (3,3',5,5'-tetraiodothyronine, T_4), and 3,3'5-triiodothyronine (T_3), are synthesized in the follicle by iodination and condensation of the tyrosyl residue in thyroglobulin. (For structures of the iodothyronines, please refer to FIG. 1.) An inactive form of triiodothyronine, iodinated at the 3,3',5' positions, named reverse T_3 (rT_3), is also synthesized in small amounts.

Thyroglobulin, a 660,000 dalton iodoglycoprotein, is synthesized in the thyroid cell. It contains three percent tyrosine by weight. Seventy percent of these tyrosyl residues are susceptible to iodination as shown by spatial studies (30). In the thyroid cell, the backbone of the polypeptide is assembled on polyribosomes on the rough endoplasmic reticulum . It is then processed and glycosylated in the smooth endoplasmic reticulum and golgi apparatus. During the process of secretion into the colloid by exocytosis, the tyrosyl residues are iodinated, forming monoiodotyrosines (MIT) and diiodotyrosines (DIT). The iodination reaction is catalyzed by peroxidase (9).

After a lag time of fourteen to sixty minutes, condensation of an iodinated hydroxyphenyl group of one peptide-linked iodotyrosine to the phenolic hydoxyl group of another peptide-linked iodotyrosine occurs (8). This results in the release of an

alanine residue and the formation of thyroxine when DIT is coupled to DIT, and T_3 or rT_3 when MIT is coupled with DIT (20)

Hormone secretion and mode of action :

The secretory process of thyroid hormones begins with the endocytosis of globules of the thyroglobulin-containing colloid. Once inside the cell, fusion with lysosomes occur with subsequent proteolysis of the glycoprotein to component amino acids, the iodinated tyrosyl residues (MIT and DIT), and the iodothyronines. The iodothyronines are subsequently released into the bloodstream while the iodinated tyrosyl residues and amino acids in the thyroid cells are recycled. This process is regulated by thyroid stimulating hormone (TSH) in a cAMP-dependent manner (9).

The fundamental action of the thyroid hormone appears to be exerted at the nuclear level. Receptors in the nuclei of target cells bind the thyroid hormones, especially T_3, with high affinity. The hormone-receptor complex activates the chromatin by an unknown mechanism, leading to the increased activities of polymerase I and II, and possibly chromatin template activity (5). This leads to enhanced synthesis of ribosomal and messenger RNA and consequently protein synthesis. This results in the influence of cellular functions.

Deiodination of thyroxine :

Several pieces of evidence have suggested that T_3 is the active form of thyroid hormone. Intracellularly T_3 is the predominant thyroid hormone (9). T_3 has been shown to bind ten to fifteen times as tightly to the thyroid hormone nuclear receptor as does T_4 (5). When deiodination of T_4 to T_3 was blocked, physiologic responses thought to be regulated by thyroid hormone were depressed or eliminated (9).

The secreted T_4/T_3 ratio (approximately 10:1) is an indication of thyroidal $T4/T_3$ ratio (9). Since T_3 is the active species, extrathyroidal deiodination of thyroxine constitutes a major pathway for thyroid hormone metabolism (for review articles, see 40,41,42). The development of sensitive and specific radio-immunoassays have greatly facilitated studies of deiodination pathways both in vivo and in vitro. The extrathyroidal conversion of thyroxine to triiodothyronine has been observed in homogenates of liver (22,40,41,42), kidney (15,22,26), thyroid (10), anterior pituitary (33), cerebral cortex and cerebellum (37,38).

Initial studies by Visser et al. (42,43) of rat liver homogenates led to the hypothesis that enzymatic deiodination of thyroxine involves a two enzyme system : a 5'-deiodinase which converts T_4 into 3,3'5-triiodothyronine (T_3) and a 5-deiodinase which converts T_4 into 3,3'5'-triiodothyronine (rT_3). Reverse T_3 is then rapidly degraded by the 5'-deiodinase to 3,3-diiodothyronine

(3,3'-T_2). However, T_3 degradation to 3,3'-T_2 by 5-deiodinase proceeds at a much slower rate (40,41,43) (See FIG. 2). Differential centrifugation of the liver homogenate revealed that majority of the deiodinating activities are recovered in the microsomal fraction (1,11,19,40,43).

The conversion of thyroxine to triiodothyronine is a reductive process, replacing an iodinium atom by hydrogen. Microsomal preparations generally require the presence of thiol-group containing compounds (e.g. dithiothreitol (DTT)) for deiodination to proceed. The addition of thiouracil derivatives, such as propylthiouracil (PTU) and 2-thiouracil, in the presence of substrate, results in a progressive and irreversible decrease in the deiodination activity in the liver and kidneys (45,46), but not in the anterior pituitary and the central nervous system (6,24). Thus we can separate the deiodinase activities into PTU-sensitive and PTU-insensitive pathways.

PTU-sensitive pathway :

The PTU-sensitive pathway is responsible for greater than sixty percent of the extrathyroidal deiodination of T_4 (9). Kinetic studies by Visser et al. (21,43,45) provided evidence for a ping-pong mechanism of deiodination (see FIG. 3). In the first half of the reaction, the enzyme interacts with T_4, leading to T_3 production and the transfer of an iodinium ion from the substrate

to a sulphydryl group on the enzyme. This results in the formation of an enzyme-sulphenyl-iodide complex (E-SI). A sulphydryl-containing cofactor (DTT in vitro) then releases the enzyme from the E-SI complex thereby freeing the enzyme for the next round of reaction (45). Thiouracil analogs inhibit this reaction by reacting with the E-SI complex and forming a mixed disulfide bond. This creates a dead-end complex, thus terminating the reaction (45, 46,52).

Although the early results of Visser (42,44) and Hoffken (18), based on studies of pH optima and kinetics, predicted the existence of distinct 5'- and 5- deiodinases, recent data by Chopra (7) and Fekkes (13) suggested that the possibilty of a single enzyme responsible for both activities cannot be ruled out. That both the tyrosyl and the phenolic ring of iodothyronines are deiodinated by the same enzyme is supported by the following recent findings :

A. Both activities have been localized to the microsomes and further fractionation has not separated their activities (1,11).

B. Both reactions are reductive and require a thiol-containing-group compound such as DTT (41,43).

C. Both activities are inhibited by thiouracil in an uncompetitive manner in the presence of a deiodinase-specific iodothyronine substrate (15,16,52).

D. 3,5-diiodothyronine, a substrate which is specifically

deiodinated by 5-deiodinase, can serve as the substrate requirement for 5'-deiodinase inhibition by thiouracil (46).

E. 5- and 5'- deiodinase activities in polyoxyethylene ether W-1 (W-1 ether) extracts of rat liver microsomal fractions co-migrate on isoelectric focusing gels (18).

F. The K_m values for the substrates undergoing 5-deiodination are equal to their K_i values for inhibition of 5'-deiodination of the other substrates and vice versa (13).

PTU-insensitive pathway :

In the central nervous system and in the anterior pituitary, intracellular T_3 is derived predominantly by local T_4 conversion (25). Silva and Leonard et al. (34) showed in vitro the presence of a T_4 to T_3 5'-deiodinating pathway in the cerebral cortex, the cerebellum, and the anterior pituitary, that was insensitive to PTU in the presence of DTT. In that same study, the liver deiodinase activity was inhibited seventy-six percent by PTU, suggesting that the PTU-insensitive pathway is quantitatively unimportant in the liver.

Visser et al. (48) reported the existence of two 5'-deiodinase activities in the cerebral cortex. One of these was specific for rT_3, required DTT as a cofactor, was sensitive to PTU, and followed the ping-pong reaction model, as in the liver. This

deiodinase predominates in euthyroid and long-term hypothyroid rats. The other activity, found in short-term hypothyroid rats, preferred T_4 to rT_3 as substrate, was insensitive to PTU, and followed sequential reaction kinetics. The relative proportion of activity of these 5'-deiodinase pathways was dependent on the thyroid status of the animal (47).

Tanaka et al. (37) reported the presence in the C.N.S. of a 5-monodeiodinase activity converting T_4 to rT_3 and T_3 to $3,3'-T_2$ that was dependent on DTT but not inhibited by PTU. This activity was localized to the synaptosomal fraction by ultracentrifugation method (10). In a later paper (38), using a $3,3'-T_2$-specific RIA, they showed that the 5'-deiodinase activity in rat brain which converts rT_3 to $3,3'-T_2$ was, however, sensitive to PTU. They suggested that the PTU-insensitive formation of $3,3'-T_2$ reported by Visser et al. (47), who measured release of ^{125}I from $^{125}I-rT_3$, was by a different pathway. Studying age related changes and different regional distribution of the enzymes, they concluded that rT_3 5'-deiodinase and T_4 or T_3 5-monodeiodinase are different enzymes (38). A definitive view of the deiodinase pathways in the C.N.S. has not been reached.

Non-enzymatic pathways :

The deiodination of thyroxine and related compounds by microsomes may also occur via non-enzymatic processes. Studies

have shown certain deiodination pathways to be accelerated with oxygen and metal ions (35,39). A NADP-dependent lipid peroxidation in a submicrosomal system (36) has also been reported. The physiological importance of these non-enzymatic pathways have not been established.

Partial purification of two deiodinase activities :

The deiodination of thyroxine by extrathyroidal tissues into T_3, rT_3, and further degardative products thus involves multiple pathways. A definitive view of the deiodination pathways has not been reached. The difficulty in arriving at a coherent picture for the extrathyrodial deiodination pathways rests in part on the failure to further purify the enzyme. The use of various detergents including cholate and deoxycholate to solubilize and purify deiodinase activities has been reported in the literature (14,26,29). This has only resulted in partial recovery of the deiodinase activity.

In our laboratory, Watkins et al. found that solubilization of rat liver microsomes by the zwitterionic detergent, CHAPS, revealed a deiodinase activity for which ^{125}I-labelled 1-(p-hydroxyphenyl)-2-guanidinoethane was a substrate (52). It was subsequently found that ^{125}I-T_4 was also deiodinated in this system. Preliminary results using labelled thyroxine revealed various breakdown products with HPLC (High Performance Liquid

Chromatography) peaks co-migrating with T_3, rT_3, T_2, and free iodide (personal communication). This finding is consistent with the products of extrathyroidal deiodination of thyroxine reported in the literature.

Using CHAPS detergent to solubilize microsomes represents an original purification procedure for deiodinase activity. Initial studies of the activity revealed a novel cofactor requirement; the deiodinase activity was stimulated up to ten fold by the presence of NADP, much less by NAD, and not at all by the reduced forms of these nucleotides, NADH and NADPH. The NADP effect was not observed with venom phosphodiesterase-treated NADP, suggesting that the enhancement was not due to a contaminant in the NADP preparation.

Partial purification of the activity by DEAE-Sephadex A25 column chromatography revealed two peaks of activity. Initial studies suggested that NADP with DTT and NADP in the presence of phospholipids may act as effectors.

As CHAPS solubilization represented a novel purification procedure for the deiodinase activity, we proceeded to relate the detection of our two activities to those reported in the literature (1,2,7,10,11). Using a commercially available T_3-specific radioimmunoassay kit, an assay system was designed to detect for T_3 as a product of T_4 metabolism in our preparation. In this study, we report the detection of a 5'-deiodinase activity in partially purified CHAPS-solubilized rat liver microsomes. On further purification, the deiodinase activity was recovered in the

"breakthrough" and "wash" fractions from DEAE-Sephadex A25 column chromatography. This activity, deiodinase A, was stimulated by the addition of NADP and DTT. A second activity was recovered upon elution of the column with 0.4 M NaCl. This second activity, deiodinase B, is enhanced by the additon of NADP in the presence of phospholipids. The production of T_3 as detected by the T_3 radioimmunoassay was less than one percent of the production of ^{125}I released by the ^{125}I-T_4 deiodinase assay. These findings are consistent with the hypothesis that thyroxine is converted to T_3 and rT_3 and subsequently into further degradative products as reported in the literature (37,38,40,41,42,43,47). The use of CHAPS to solubilize microsomal deiodinase activities may be used as a mean to further purify and characterize the identity of the various deiodinases present in extrathyroidal tissues.

Methods and Materials

Materials and miscellaneous methods :

Male Osborne-Mendel rats (6-8 weeks old) were obtained from the NIH breeding colony and were fed ad libitum; NAD, NADP, NADH, NADPH, and T_2 (diiodothyronine) were obtained from Sigma; T_4 (thyroxine), T_3 (3,5,3'-triiodothyronine), and rT_3 (3,3'5'-triiodothyronine) were obtained from Calbiochem; ^{125}I-T_4 and ^{125}I-T_3 were obtained from New England Nuclear; T_3 radioimmunoassay kits were from Amersham; DTT (dithiothreitol), CHAPS (3-((3-cholamidopropyl)-dimethylammonio)-1-propane sulfonate), MES (2-(N-morpholino)ethanesulfonic acid), and Tris-HCl were obtained from Bethesda Research Laboratories; Phospholipids (Cat. # 6202) were obtained from Pharmacia L-P Biochemical; DEAE-Sephadex A25 was obtained from Pharmacia; Dowex AG 50-X8 was obtained from Bio-Rad. Protein analysis was determined by the method of Lowry et al (28).

Partial purification of the two deiodinase activities from the rat liver :

A. Preparation of crude microsomes.

Livers were homogenized (1:3, w/v) in 0.25 M sucrose containing 25 mM Tris-HCl, pH 7.4, with a Potter-Elvehjem homogenizer. Homogenates

were centrifuged at 25 000 x g for 25 min. After removal of the floating fatty layer, the supernatant was further centrifuged for 60 min. at 105 00 x g to sediment the microsomes. This microsomal pellet was then resuspended in 0.4 volumes of homogenization buffer (0.25 M sucrose in 25 mM Tris-HCl, pH 7.4).

B. Detergent solubilization.

Suspensions of microsomes were diluted 1:1 with homogenization buffer and CHAPS was added to a final CHAPS concentration of 0.8%. After 10 min. on ice, the mixture was again centrifuged at 105 000 x g for 60 min.

C. Acid treatment.

The pH of the CHAPS-soluble fraction (105 000 x g supernatant) was lowered by the addition of 0.1 volume of 1.0 M NaAcetate, pH 4.5. After 15 min. on ice, precipitated material was removed by centrifugation at 7 000 x g for 10 min. The supernatant was then removed and the pH adjusted to 7.0-7.5 by the addition of 1.0 M Tris-base. This will be referred to as the pH 4.5 supernatant.

D. DEAE-Sephadex A25 chromatography.

Small Column

A column (0.8 x 5.0 cm) of DEAE-Sephadex A25 was equilibrated with 25 mM Tris-HCl, pH 7.5, containing 0.8% CHAPS. The pH 4.5 supernatant was diluted 1:5 with this buffer. 2.0 ml was applied to the column. The sample was then washed in with 3.0 ml of buffer and collected as

the "breakthrough". The column was further washed with three 5.0 ml portions of buffer. These are the "wash" fractions. Finally the column was eluted with successive 1.0 ml aliquots of buffer containing 0.4 M NaCl and ovalbumin (1.0 mg/ml) These are the "eluate" fractions.

Large Column

Volumes in the large column (2.5 x 3.0 cm) DEAE-Sephadex 25 were increased 1:5 volumes proportionally from the small column.

E. Dialysis.

To decrease the concentration of CHAPS, the enzyme preparations were dialyzed overnight against 7 volumes of 25 mM Tris-HCl, pH 7.5, at 4° C.

Preparation of phospholipids :

Phospholipids (Cat. # 6202), consisting of 12 uM phosphatidyl choline, 12 uM sphingomyelin, 12 uM phosphatidyl ethanolamine, 5.2 uM phosphatidyl serine, 0.4 uM cardiolipin, and 0.4 uM phosphatidyl inositol, were dissolved in 2.0 ml chloroform and stored at 20° C. Aliquots of 100 ul of phospholipids were prepared fresh for each assay by transferring the phospholipids to a glass tube, air dried with nitrogen gas at room temperature, and suspended in 600 ul of 50 mM MES, pH 6.0, and ovalbumin (1.0 mg/ml).

^{125}I-T_4 deiodinase assay :

Assays contained, in total volume of 0.1 ml, 50 mM MES, pH 6.0, ovalbumin (1.0mg/ml), 25 nM ^{125}I-T_4 (approximately 100 000 dpm/assay), 10 ul enzyme, and other additions as described for each experiment. After incubation for 10 min, at 30° C, the reaction was terminated by the addition of 1.0 ml of 10% acetic acid. Released ^{125}I was separated from the substrate by loading the entire sample on a 1.0 ml column of Dowex AG 50-X8 (equilibrated with 10% acetic acid) and washing the column with five 1.0 ml portions of 10% acetic acid. Radioactivity was then measured with a Beckman Auto-gamma counter. Under these conditions, greater than 97% of ^{125}I-T_4 and ^{125}I-T_3 remain bounded to the column whereas ^{125}I was quantitatively eluted.

T_3 radioimmunoassay:

To determine the amount of T_3 formed during the deiodinase asssays, incubation mixes were analyzed using a commercial T_3 radioimmunoassay kit obtained from Amersham. Incubations contained in a total volume of 0.25 ml, 50 mM MES, pH 6.0, ovalbumin (1.0 mg/ml), 1 uM T_4, 0.2 ml enzyme, and other additions as described for each experiment. After 20 min. at 30° C, the reaction was terminated by transferring a 0.2 ml aliquot to a microfuge tube containing 0.4 ml ice-cold ethanol.

The tube was then spun for 15 min. and duplicate 50 ul aliquots of the supernatant were removed for radioimmunoassay. Samples were mixed with 0.5 ml of ^{125}I-T_3 solution and 0.5 ml anti-T_3 antibody solution. After incubation for 60 min, at 37° C, the tubes were centrifuged at 1 500 x g for 15 min. at room temperature. Radioactivity in the pellet was then quantitated in a Beckman Auto-gammma counter.

Results

Characterization of the radioimmunoassay :

A commercially available T_3-specific radioimmunoassay kit was used to detect 3,5,3'-triiodothyronine in our assay system. In Figure 4, a semilog plot of a typical concentration curve is shown. This was generated under conditions typical of our unknown samples containing 50 mM MES, pH 6.0, ovalbumin (1.0 mg/ml), 3 mM NADP, and 5 mM DTT. This curve is readily reproducible and is generated independently for each experiment.

The specificity of a radioimmunoassay is critical to its usefulness. Therefore, we first checked the assay for cross-reactivity with other iodothyronines. Data supplied with the radioimmunassay kit stated that cross-reactivity of the anti-T_3 antibody was, by weight, 0.8% for T_2, less than 0.03% for rT_3, and less than 0.3% for T_4. In Table I, using standards obtained from Sigma and Calbiochem in our buffer system, cross-reactivities for T_2, rT_3, and T_4 were 2.2%, 0.2% and 1.1% respectively.

Detection of rat liver deiodinase activity by ^{125}I-T_4 :

Deiodinase activity in crude rat liver homogenates was detected

as release of free ^{125}I from ^{125}I-T_4 (FIG. 5, (H)). However, this activity was not significantly increased when assays contained NADP. NADP-stimulated T_4 deiodinase activity was partially purified from homogenates as described in Methods. Previous results of differential centrifugation of rat liver homogenates showed that most of the NADP-stimulated deiodinase activity was in the microsomal fraction (data not shown). Activity of the microsomal fraction was found to be increased ten-fold when NADP was present (FIG. 5, (M)).

Treatment of the microsomal preparation with 0.8% CHAPS effectively solubilized the deiodinase activity (FIG. 5, (C)). Acidification of the detergent-solubilized enzyme preparation with 0.1 vol NaAcetate, pH 4.5, caused the precipitaion of much of the microsomal protein. The deiodinase activity, however, remained in the supernatant. The pH 4.5 supernatant, which was subsequently neutralized to pH 7.0-7.5, exhibited higher total activity than either the microsomes or the CHAPS-solubilized fractions, suggesting that some inhibitory material had been removed (FIG. 5, (S)).

To further purify the deiodinase activity of the pH 4.5 supernatant, the preparation was subjected to chromatography on DEAE-Sephadex A25. As shown in Figure 6A, most of the NADP-dependent activity was present in the "breakthrough" (BT) and "wash" (W1,W2,W3) fractions. This activity, which we will refer to as deiodinase A, was stimulated by NADP or DTT. The presence of both effectors

synergistically increased activity (FIG. 6B). A second and much smaller activity peak eluted from the column with 0.4 M NaCl. This activity, deiodinase B, was not affected by DTT (FIG. 6B).

Since many membrane associated enzymes require phospholipids (26), column fractions were assayed in the presence of phospholipids (for preparation, see Methods). As shown in Figure 7A and 7B, phospholipids increased the activity of deiodinase B. When NADP was present in addition to phospholipids, further enhancement of the activity in deiodinase B was observed (FIG. 7B). The effect of NADP on deiodinase A activity was more significant than that of phospholipids. These results suggested that there are two deiodinase activities in the CHAPS-solubilized fraction of rat liver microsomes.

Detection of rat liver deiodinase activity by T_3 radioimmunoassay :

Following the methodology described in the original report by Visser et al. (40) for the detection of T_3 in rat liver homogenates by T_3 radioimmunoassay, a modified T_3 detection assay system was set up.

Rat liver was homogenized in 3 volumes of 0.25 M sucrose in 25 mM Tris-HCl, pH 7.4, buffer, and centrifuged at 2,000 x g for 5 min. Using the supernatant, T_3 was detected when thyroxine (final concentration = 1 uM) was added to the standard reaction mixture as described in Methods with and without the addition of 3 mM NADP and

5 mM DTT (FIG. 8, (SUP 1)). The amount of T_3 detected was greater when NADP and DTT were present.

The amount of T_3 detected by the radioimmunoassay was in the picograms range. The final concentration of T_4 introduced into the assay system was 1 uM. By cross-reactivity studies, 1.1% of T_4 may be detectable as T_3 under our assay conditions. The addition of the same amount of T_4 to our control assay (buffer without enzyme) thus serves as a baseline for cross-reactivity and the picograms of T_3 detected above control corresponds to T_3 generated. The values in the following graphs have been corrected for with subtractions of the controls and therefore reflect T_3 generated.

The homogenate supernatant was then subjected to the same partial purification process as for the enzyme preparation used in the ^{125}I-T_4 deiodinase assay (see Methods). Deiodinase activity, as measured by T_3 produced, was observed in the supernatant after centrifugation at 25,000 x g (FIG. 8. (SUP2)). The supernatant was then respun at 105,000 x g. Most of the deiodinase activity was recovered in the microsomes (FIG. 8. (SUP3) and (M)). The presence of NADP and DTT increased the conversion of thyroxine to T_3.

The addition of 0.8% CHAPS and centrifugation of enzyme preparation at 105,000 x g resulted in a CHAPS-soluble fraction and a CHAPS-pellet. Deiodinase activities were present in both (FIG. 9 (C-SOL) and (C-PEL)). Acid treatment of the CHAPS-soluble fraction with NaAcetate resulted in a soluble fraction and precipitate.

Neutralization and resuspension of respective preparations revealed less activity than the pretreatment CHAPS-soluble fraction (FIG. 9 (4.5-SOL) and (4.5-PEL)). The majority of the activity remained in the soluble fraction. As shown, the deiodinase activity was detectable only with the addition of NADP, DTT and phospholipids.

DEAE-Sephadex A25 column chromatography was used to partially purify the activity identified in the pH 4.5 supernatant. As shown in Table II, no deiodinase activity as measured by T_3 radioimmunoassay was observed in deiodinase A. However T_3 was detected when microsome and pH 4.5 supernatant were tested for deiodinase activity. The activity in microsomes was about 2 times that of the pH 4.5 supernatant.

Using the same deiodinase A preparation as above, $^{125}I\text{-}T_4$ was used as substrate to test for deiodinase activity using the $^{125}I\text{-}T_4$ deiodinase assay per Methods. Using this assay, 180 and 11.8 picograms of $^{125}I\text{-}T_4$ were deiodinated by the enzyme preparation in the presence and absence of NADP and DTT respectively (data not shown). The results suggested that there may be inhibitory factor(s) present in the T_3 assay but not in the $^{125}I\text{-}T_4$ deiodinase assay.

A set of experiments were run to eliminate possible inhibitory factor(s). Figure 10 shows the inhibition of the $^{125}I\text{-}T_4$ deiodinase assay by increasing concentration of CHAPS. When the concentration of CHAPS exceeds 0.2%, complete inhibition of the deiodinase activity as measured by the release of free ^{125}I was observed.

The final concentration of CHAPS in the enzyme fractions from chromatography were approximately 0.8%. In the ^{125}I-T_4 deiodinase assay, 10 ul of enzyme was used in a total reaction volume of 100 ul, resulting in a final concentration of 0.08% CHAPS. In the T_3 assay, 200 ul of the 250 ul total volume of reaction mixes was enzyme preparation. This resulted in a final concentration of 0.64% CHAPS, well into the inhibition zone depicted in Figure 10. To reduce the CHAPS concentration, overnight dialysis in 7 volumes of 25 mM Tris-HCl, pH 7.4, at 4° C was performed.

The recovery of the deiodinase activity by overnight dialysis as measured by the T_3 radioimmunoassay is shown in Table III. Although there is some loss of activity as the enzyme was purified from microsomes, deiodinase activity was recoverable in the dialyzed enzyme preparations corresponding to deiodinase A. This 5'-deiodianse activity was readily stimulated by the addition of NADP and DTT.

The amount of enzyme available using the 5 ml DEAE column was insufficient for our protocol, In Figure 11 and 12, the comparison of the fractional activities between the large column (5 x small column) and small column, measured by ^{125}I-T_4 deiodinase assay, is shown. The elution profile of the large column correlated well with the small column. This enabled us to increase the volume of enzyme to be chromatograhed proportionally by 5 times and the pooling of the "breakthrough" and "wash" fractions as deiodinase A, and the "elution" fractions (E3-E6) as deiodinase B.

Table IV demonstrates that the increase of column size and pooling of the enzyme fractions preserved the activity in deiodinase A as measured by T_3 radioimmunoassay. The activity was synergistically enhanced by the addition of NADP and DTT.
The amount of T_3 generated by deiodinase B was not significant.

Various concentrations of T_4 were then studied with a constant concentration of enzyme as shown in Table V. At 0.1 uM T_4, T_3 production was below detectable levels. Both at 0.5 uM and at 1.0 uM T_4, deiodinase A activity can be demonstrated which is stimulated by NADP and DTT. Substrate concentration does not appear to be rate-limiting in our assay system. With and without the addition of NADP and phospholipids, only minimal amount of T_3 was detectable in the deiodinase B fraction.

DISCUSSION

The presence of deiodinase activity in rat microsomes has been well documented (11,29,40,41,42,43). Using ^{125}I-T_4 as substrate, deiodinase activity was detected using Dowex AG 50-X8 anion exchange chromato- graphy by the generation of free ^{125}I. Purification of microsomal activity by CHAPS solubilization, by acidification, and by DEAE- Sephadex A25 column chromatography resulted in the separation of the activity into two deiodinase activities. The first peak of activity (deiodinase A), which eluted as the "breakthrough" and "wash" fractions, was stimulated by the addition of NADP and DTT. The second peak of activity (deiodinase B), eluted with 0.4 M NaCl and ovalbumin (1.0 mg/ml), was stimulated by NADP in the presence of phospholipids. Using a T_3-specific radioimmunoassay, the generation of T_3 was studied in both deiodinase activities. The addition of NADP and DTT markedly stimulated the production of T_3 by the deiodinase activity A. This finding supported the results of the T_4 deiodinase assay. Much less T_3 was detectable by radioimmunoassay in deiodinase B incubated with T_4 with and without NADP and phospholipids. The presence of deiodinase B activity was however shown by the production of free ^{125}I from ^{125}I-T_4 deiodinase assay.

The amount of ^{125}I released from ^{125}I-T_4 by either deiodinase A or B was significantly greater than the amount of T_3 formed; the amount of T_3 was less than 1% of the total free ^{125}I released. There

are a few possible reasons for this discrepancy.

HPLC analysis of the products of both deiodinases showed that the major product was free ^{125}I with much smaller amounts of radioactivity corresponding to T_3, rT_3, and T_2. Using our assay, partially purified microsomal deiodinases A and B are capable of converting thyroxine to T_3 and subsequently to further degradative products. Therefore, only a small fraction of degraded T_4 accumulates as T_3. This finding is consistent with the hypothesis that thyroxine is converted to T_3 and rT_3 and subsequently into further degradative products as reported in the literature (37,38,40,41,42,43,47).

The ^{125}I-T_4 used in these studies contains a single radioactive iodide in the outer ring of thyroxine. As such, HPLC detection of the breakdown products of thyroxine is possible only for deiodination of the inner ring, the unlabelled outer ring iodide, and ether-link cleavage. If the labelled iodide is cleaved, only free ^{125}I will be detected. Preferential deiodination of the labelled iodide may occur due to a kinetic isotope effect. This may also contribute to the discrepancy between the free ^{125}I and T_3 detected.

Finally, the presence of an ether-link cleavage activity has been reported by Balsam et al (18). The labelled ether-link cleavage product may elute from Dowex chromatography under the same conditions that free ^{125}I elutes. This can result in the false detection of the the ether-link cleavage product as "free ^{125}I" and contribute further to the discrepancy.

The observation that significant T_4 was converted to T_3 as detected by T_3 radioimmunoassay by deiodinase A but not by deiodinase B suggests that either 5'-deiodinase activity is absent in deiodinase B or that under our assay condition, T_3 produced was rapidly degraded into further breakdown products of thyroxine and does not accumulate in sufficient quantity for detection by the radioimmunoasssay. Inhibition of T_3 deiodination and kinetics studies are needed to verify the existence of 5'-deiodinase activity in deiodinase B.

CHAPS solubilization of micrsosomes and subsequent DEAE-Sephadex A25 column chromatography represents a novel purification procedure. Deiodinase activities A and B may be unique; the activities reported here are stimulated by NADP, much less by NAD, and not at all by the reduced forms, NADH and NADPH.

In a review of the literature, there has been no NADP-dependent or enhanced 5'-deiodinase reported. Visser reported NAD, but not NADH, NADP, or NADPH, to be stimulatory for the 5'-deiodinase in rat liver microsomes (40,41,43). The association of deiodination with NADPH-dependent lipid peroxidation in a submicrosomal system has also been reported (36). In fasting rats, the decreased T_4 to T_3 conversion was reported to be due to a missing factor in the cytosol, correctable by the addition of NADPH (3,23). Visser proposed that the lower glucose availability state in fasting leads to depresssed hexose monophosphate shunt pathway activity and thus a low NADPH

level (43). This relationship between the hexose monophosphate shunt pathway and T_4 to T_3 conversion was subsequently demonstrated by Sato (31). The proposed mechanism suggested that NADPH restores cytosolic glutathione, an in vivo thiol cofactor for the enzyme. In rat liver microsomes of hypothyroid rats, the defect lies in the 5'-deiodinase. Accordingly, NADPH replacement has been shown to be ineffective in correcting the decreased activity (2)

One possibile explanation for the NADP requirement in deiodinase activities A and B may be that the increase in the amount of T_4 deiodinated in presence of NADP resulted from NADP enhancement of deiodinases other than the 5'-deiodinase, thus leading to an increased turnover rate of T_3 as exemplified by the high rate of ^{125}I-T_4 deiodination but not of T_3 accumulation by deiodinase B activity. The other possible explanation is that the deiodinase A and B activities reported here represent novel activities recovered by CHAPS solubilization of microsomes. Further correlation with the deiodinase activitites reported in the literature would involve testing for PTU-sensitivity in deiodinase activities A and B, and comparison of kinetic properties.

The use of cholate and a mixture of nonionic Brij detergents to solubilize rat liver and kidney microsomes resulting in partial inactivation of the enzyme has been reported(12,26). The partial delipidation involved in solubilization may be a factor in the incomplete recovery of activity. The addition of phospholipids

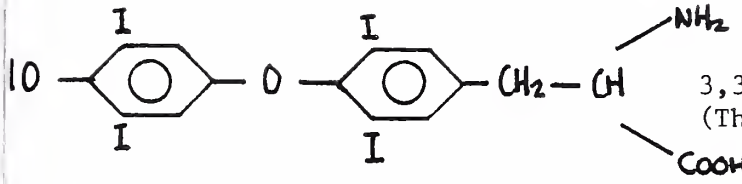
have been reportedto improve deiodinase activity (26). The addition of a phospholipids mixture with its composition as described per Methods resulted in the increase of deiodinase B activity detected by the release of ^{125}I from ^{125}I-T_4. Further work on the identification of specific phospholipid requirements may improve the T_3 yield by deiodinase B thus allowing for further characterization of the activity.

The presence of detergents have also been implicated for decreased deiodinase activity (12). The use of CHAPS detergent in concentration higher than 0.2 % resulted in the complete inactivation of the deiodinase activity as measured by release of free ^{125}I. Thus overnight dialysis of the enzyme prep to decrease the concentration of CHAPS to 0.1% was undertaken with subsequent recovery of some T_4 to T_3 conversion activity by deiodinase A. The possibility that enzyme denaturation and precipitation from overnight dialysis and decreased CHAPS concentration caused the decrease in activity cannot be excluded and thus needs further evaluation.

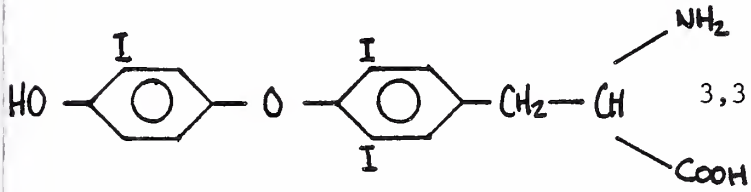
In summary, CHAPS solubilization, acidification, and subsequent DEAE-Sephadex A25 column chromatography of rat liver microsomes resulted in the separation of two deiodinase activities as measured by the release of free ^{125}I from ^{125}I-T_4.Characterization of the activities revealed a stimulatory effect of NADP on both activities. The addition of DTT with NADP to deiodinase A showed

synergistically increased deiodinase activity. Similarly, the addition of phospholipids with NADP enhanced the activity of deiodinase B. Using a T_3-specific radioimmunoassay, conversion of T_4 to T_3 was detected in deiodinase A preparations, enhanced by the presence of NADP and DTT. Only minimal amount of T_3 was detectable in deiodinase B preparations. The amount of T_3 detected was less than 1% of the amount of free ^{125}I released from ^{125}I-T_4. This is consistent with the hypothesis that thyroxine is converted to T_3 and rT_3 and subsequently into further degradative products as suggested by Watkins' HPLC data (publication in preparation) and in the literature (37,38,40,41,42,43,47). The results presented here provide a basis for further studies on CHAPS solubilization of microsomes as a mean of deiodinase purification to characterize the identity of the various deiodinases present in extrathyroidal tissue.

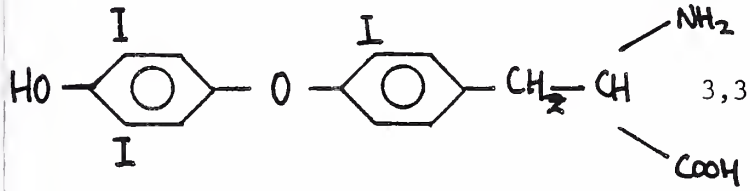
IODOTHYRONINES



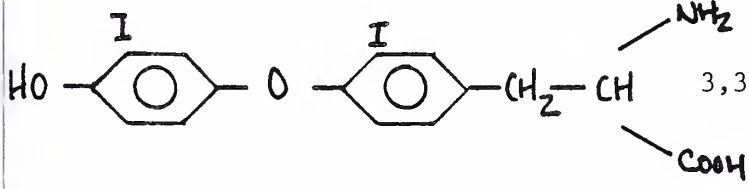
3,3'5,5'-TETRAIODOTHYRONINE
(Thyroxine, T_4)



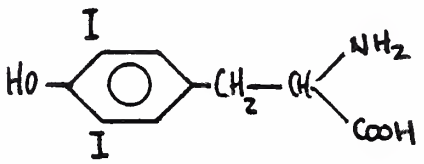
3,3'5-TRIIODOTHYRONINE (T_3)



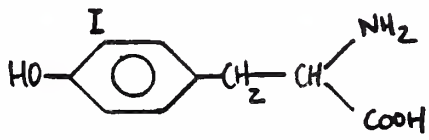
3,3'5'-TRIIODOTHYRONINE (rT_3)



3,3'-DIIODOTHYRONINE (T_2)



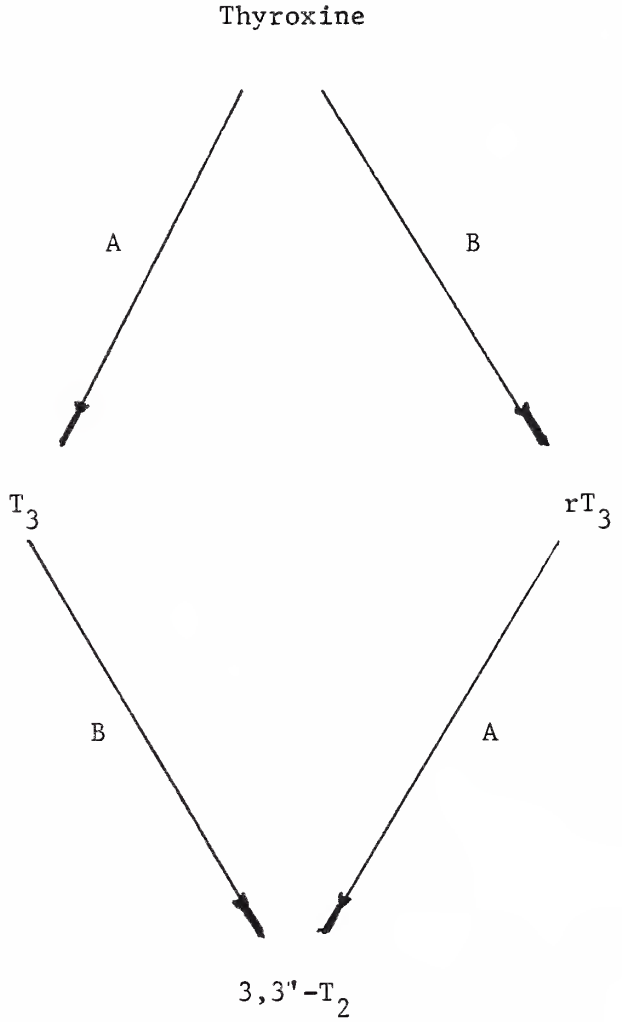
MONOIODOTYROSINE (MIT)



DIIODOTYROSINE (DIT)

FIG. 1

DEIODINATION PATHWAY



A = 5'-DEIODINASE
B = 5-DEIODINASE

FIG. 2

PTU-SENSITIVE PATHWAY

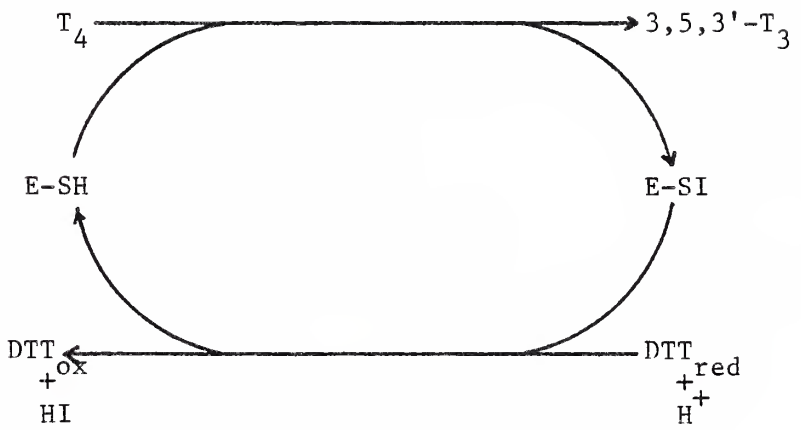


FIG. 3

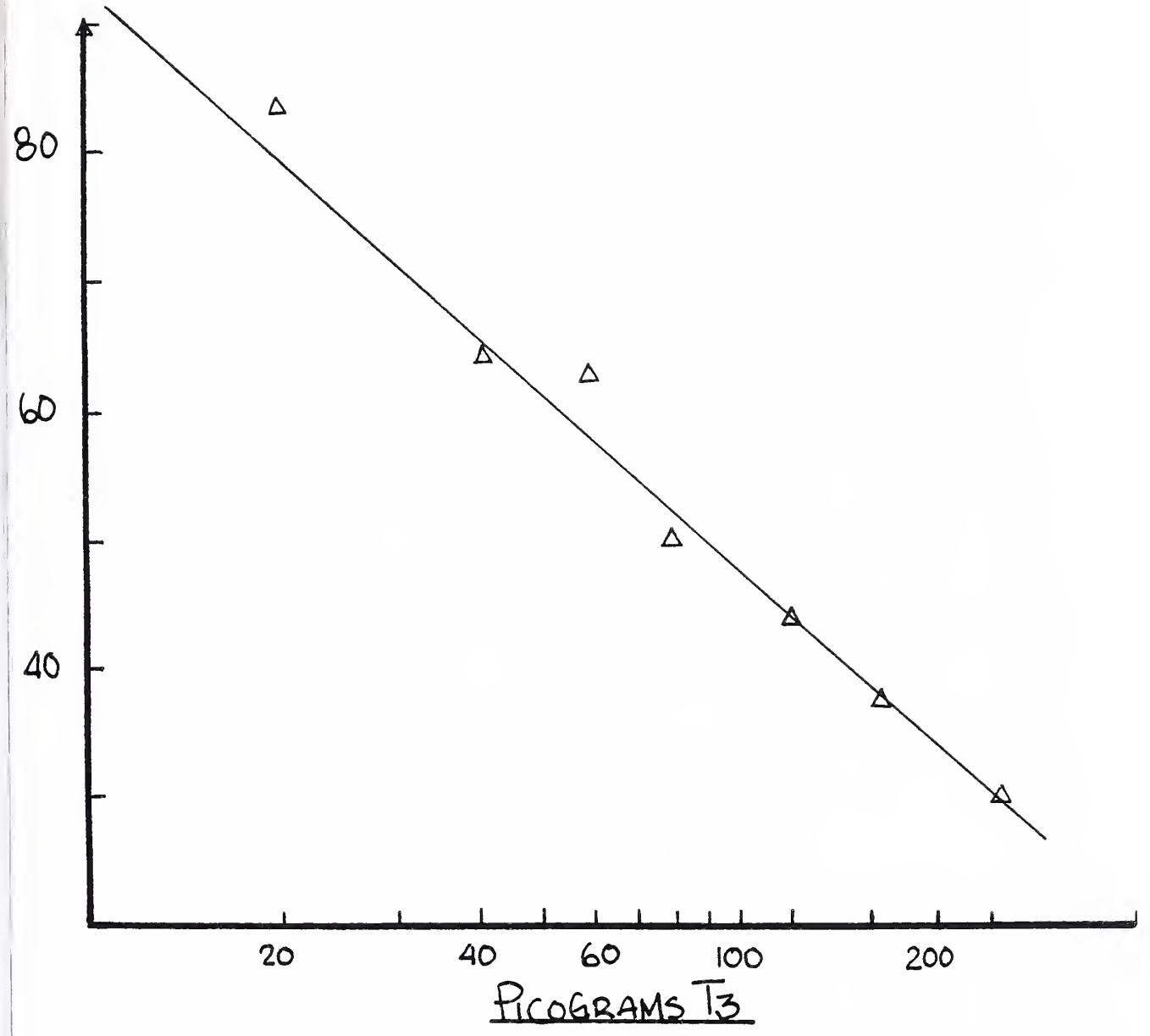


FIG. 4. Standard T_3 RIA curve using 1:1 500 ul aliquots of Amerlex^{TM} ^{125}I-T_3 to anti-T_3 antibody with 50 ul of increasing concentration of unlabelled T_3 in 50 mM MES, pH6.0, and ovalbumin (1.0 mg/ml), incubated at 37° C for 60 min.

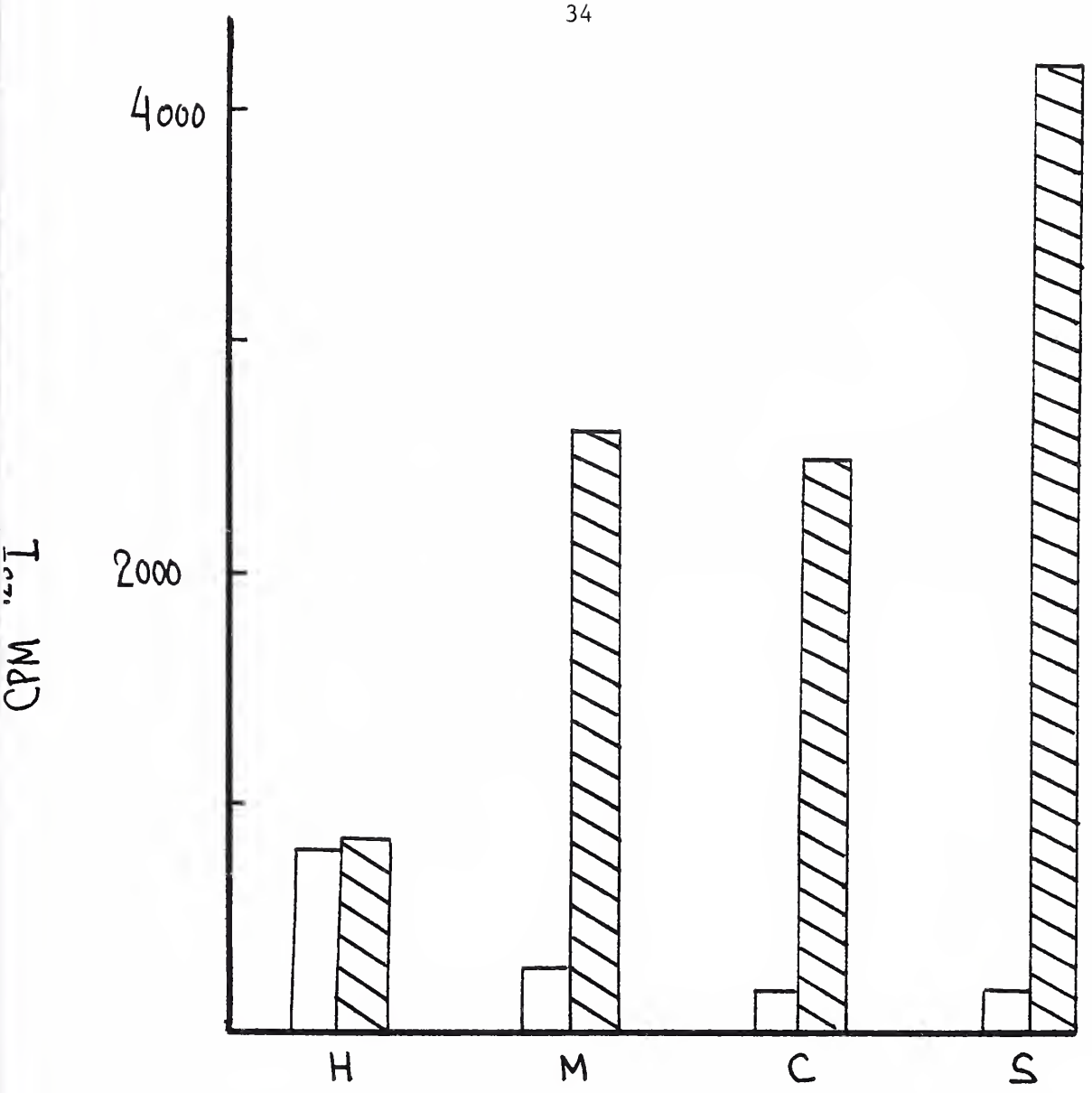


FIG. 5. Deiodinase activity, as measured by CPM of free ^{125}I (with controls subtracted), in the (H) homogenate fraction, (M) microsomes, (C) CHAPS-solubilized microsomes, and (S) pH 4.5 supernatant (CHAPS-solubilized microsomes treated with 0.1 vol of NaAcetate, pH 4.5 and neutralized with Tris-base to pH 7-7.5 as per Methods and Materials) is shown. Standard reaction mixes, in a total volume of 0.1 ml, containing 50 mM MES, pH 6.0, ovalbumin (1.0 mg/ml), 25 nM ^{125}I-T_4, and 10 ul of the indicated enzyme prep (H,M,C,orS) with ▨ and without ▭ 3 mM NADP, were incubated at 30° C for 10 min.

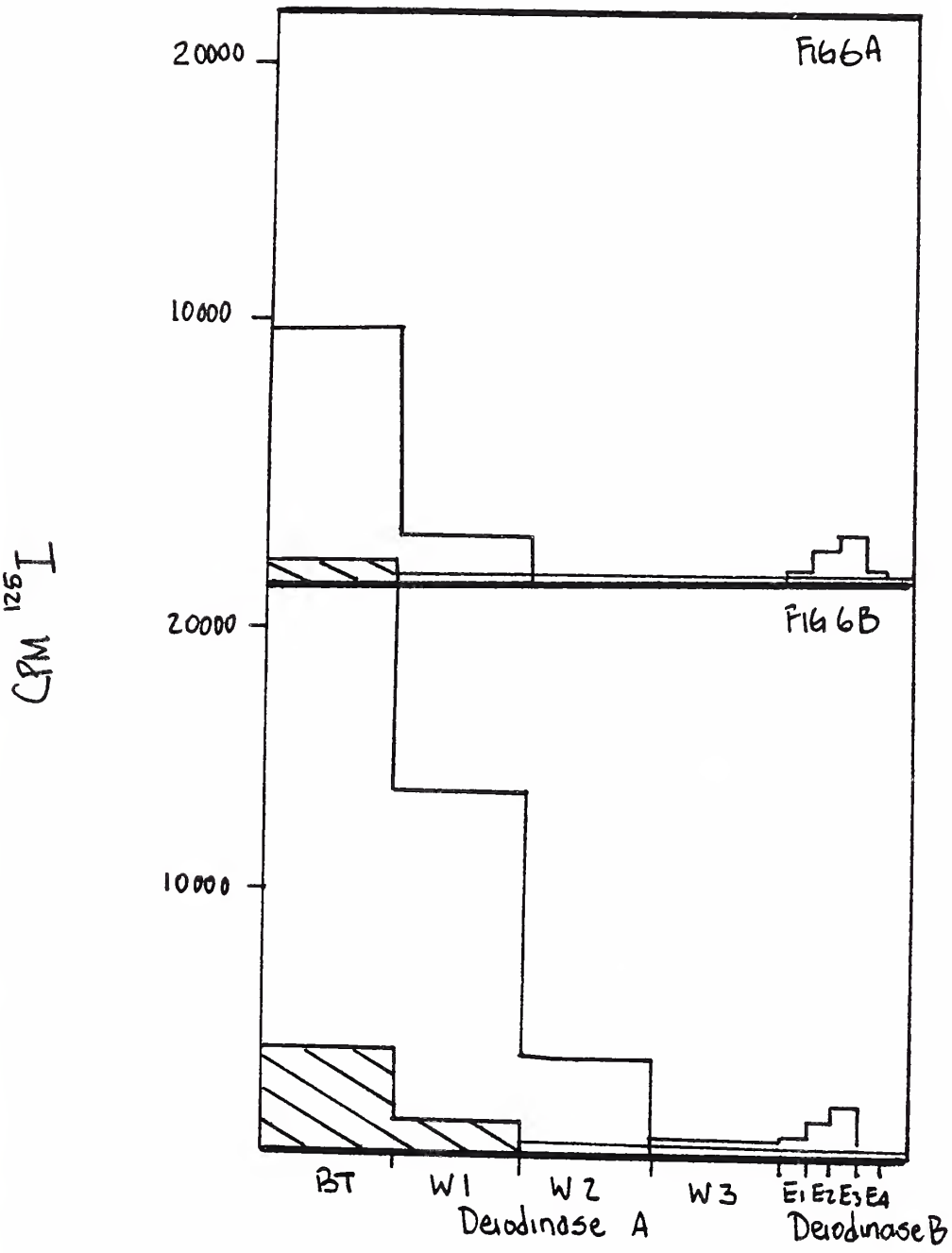


FIG. 6. DEAE-Sephadex chromatography of the neutralized pH 4.5 supernatant. Deiodinase activity in the (BT) "breakthrough" and (W1,W2,W3) "washes" (3 X 5ml aliquots of 25 mM Tris-HCl, pH 7.5, 0.8% CHAPS), designated as deiodinase A, and in the (E1,E2, E3,E4) "eluates" (4 X 1 ml aliquots of 25 mM Tris-HCl, pH 7.5, 0.8% CHAPS, 0.4 M NaCl and ovalbumin (1.0 mg/ml)), designated as deiodinase B, is demonstrated by ^{125}I-T_4 deiodinase assay. Standard reaction mixes and assay conditions as per Methods and Materials with (6B) and without (6A) 5mM of DTT, and in the presence ▭ and absence ▨ of 3mM NADP.

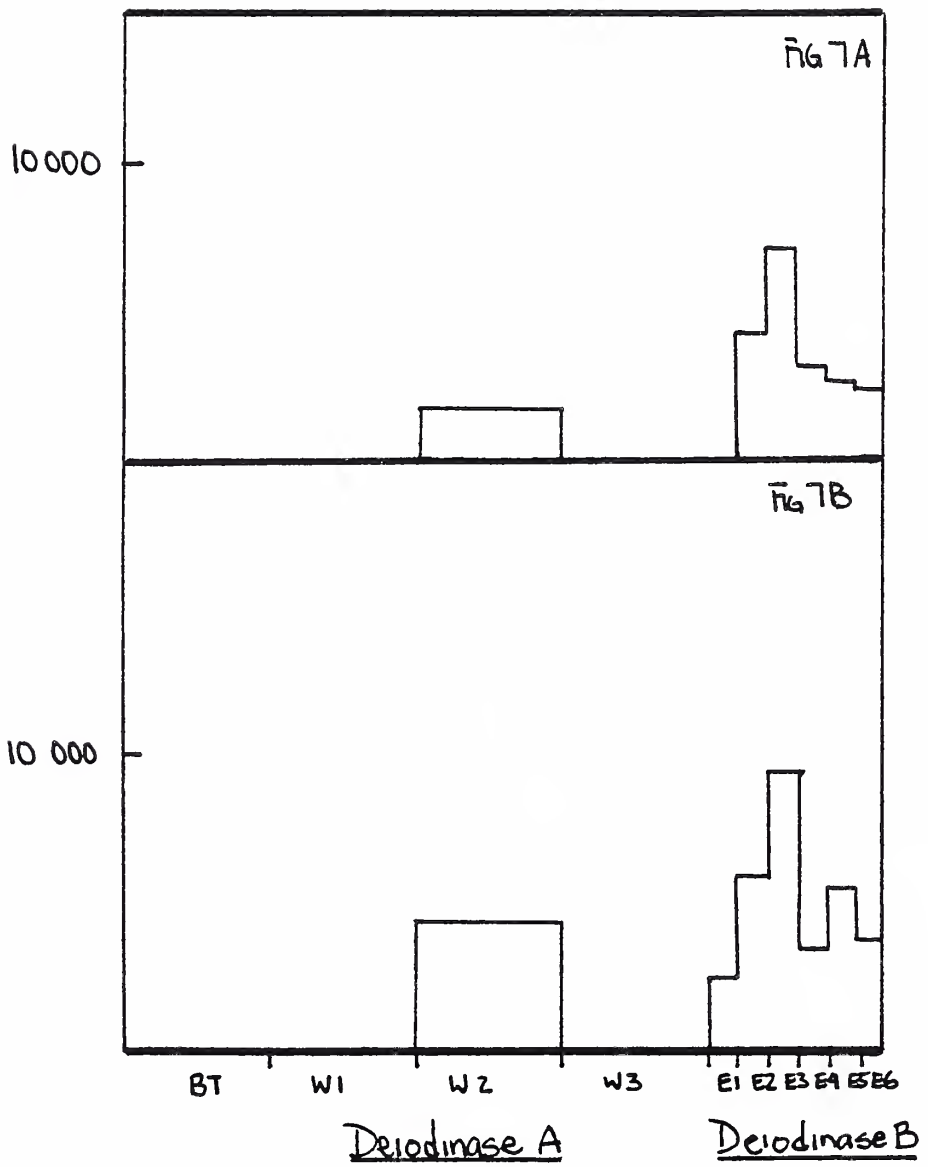


FIG. 7. DEAE-Sephadex chromatography of the neutralized pH 4.5 supernatant. Activity in deiodinase A and deiodinase B is demonstrated by $^{125}I\text{-}T_4$ deiodinase assay. Standard reaction mixes and assay conditions as per Methods and Materials with the addition of 10 ul of phospholipids with (7B) and without (7A) 3 mM NADP.

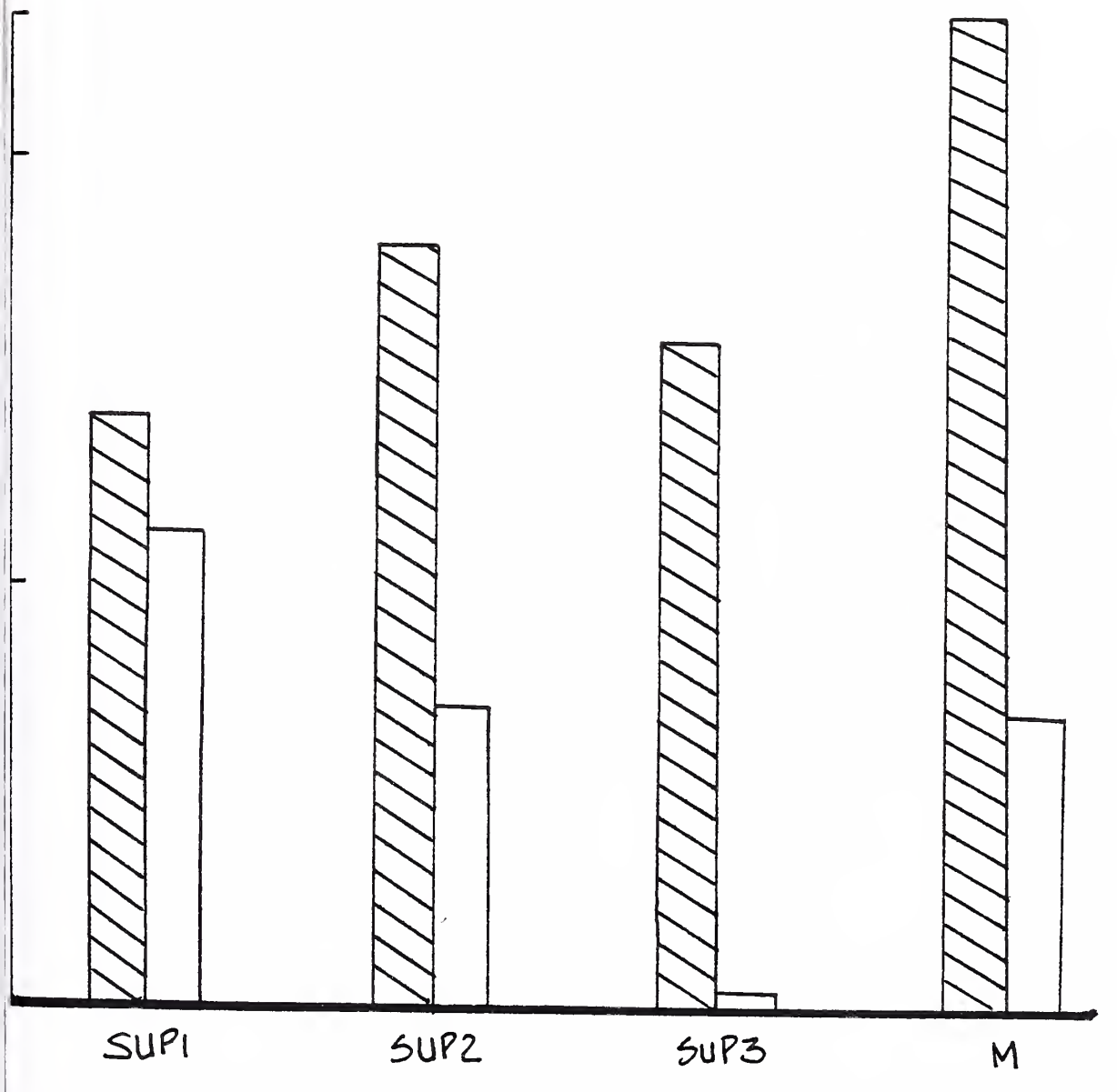


FIG. 8. Deiodinase activity, as measured by picograms of T_3 produced, in the supernatant of liver homogenate centrifuged at 2 000 x g (SUP 1), then at 25 000 x g (SUP 2), and finally at 105 000 x g (SUP 3), and in the microsomes (M), is shown. Standard reaction mixes, in total volumes of 0.25 ml, containing 50 mM MES, pH 6.0, ovalbumin (1.0 mg/ml), 1 uM T_4, 0.2 ml of the indicated enzyme prep, with ▨ and without ▭ 3 mM NADP and 5 mM DTT, were incubated at 30° C for 25 min. Reactions were then terminated by transferring a 0.2 ml aliquot to a microfuge tube containing 0.4 ml ice cold ethanol. T_3 RIA was then performed as per Methods and Materials.

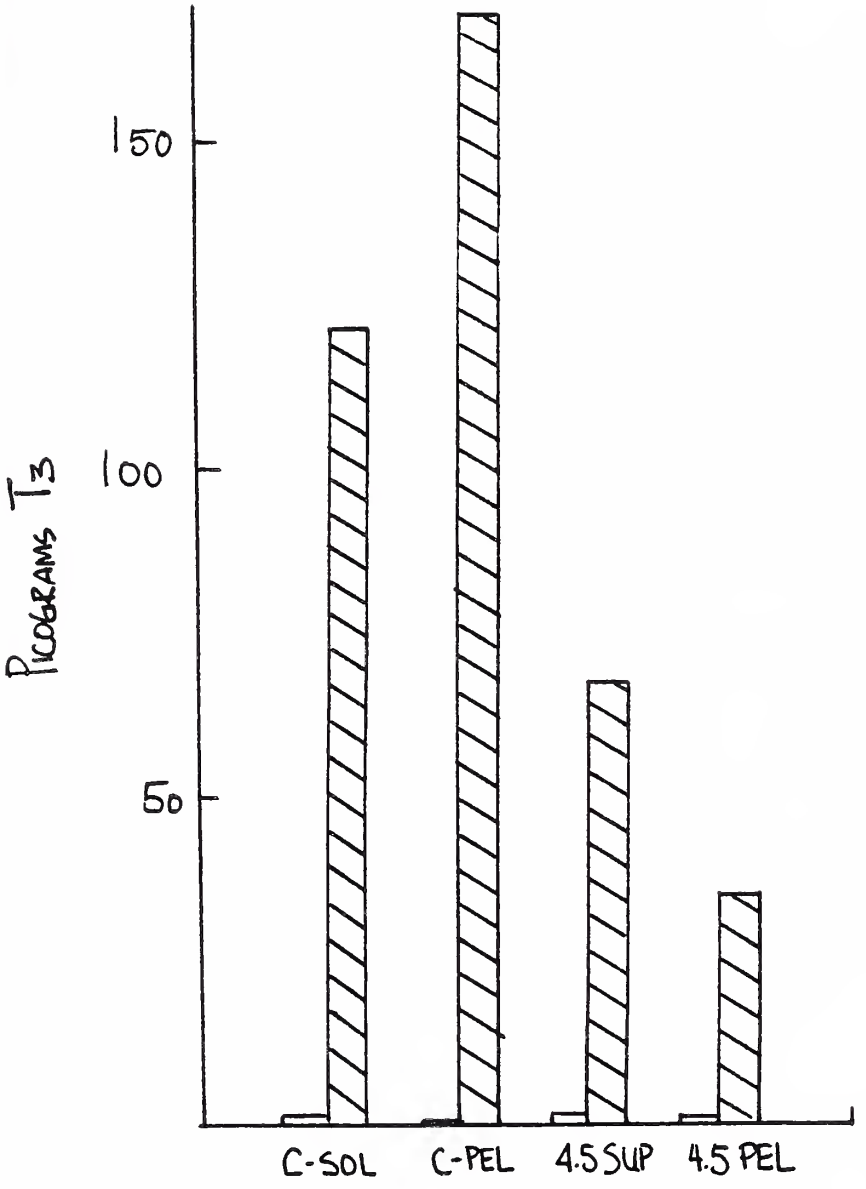


FIG. 9. Microsomes, treated with 0.8% CHAPS, centrifuged at 105 000 x g, produced a CHAPS-soluble (C-SOL) and a CHAPS-insoluble pellet (C-PEL). The pH of a portion of the CHAPS-soluble fraction was then lowered by the addition of 0.1 vol NaAcetate, pH 4.5, kept for 15 min. in ice, and centrifuged at 7 000 x g for 10 min, separating the prep into a pH 4.5 supernatant and a precipitate. The supernatant was neutralized with Tris-base to pH 7-7.5 (4.5 SUP) and the pellet was resuspended in 5 vols of 25 mM Tris-HCl, pH 7.5 (4.5 PEL). The deiodinase activity, measured by picograms of T_3 produced, is shown. Standard reaction mixes, with ▨ and without ▭ 3 mM NADP, 5 mM DTT, and 10 ul phospholipids, and assay conditions are identical to those as per Methods and Materials.

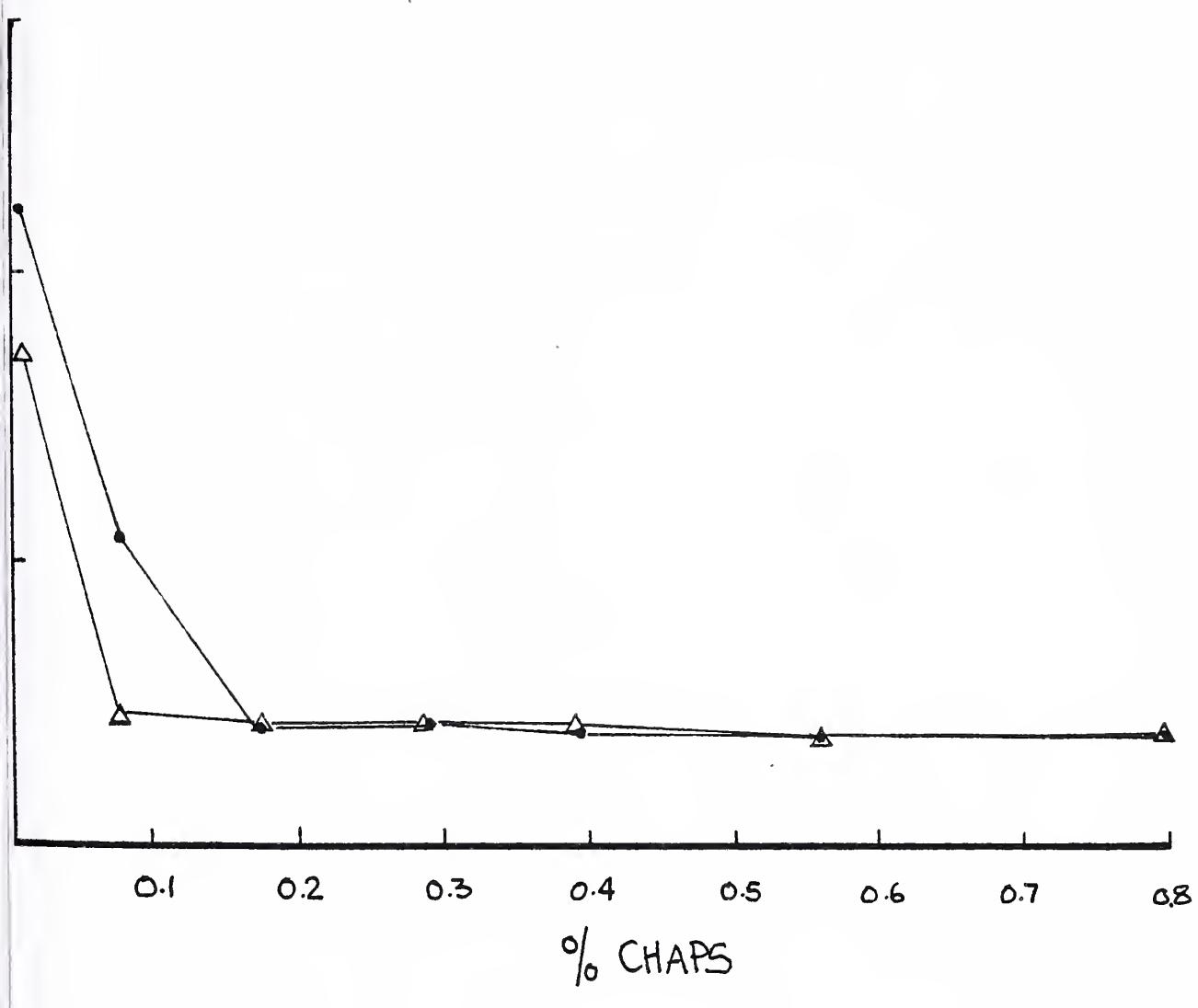


FIG. 10. The effect of increasing concentration of CHAPS on deiodinase activity, measured by $^{125}I\text{-}T_4$ deiodinase assay (as per Methods and Materials), with ●——● and without △——△ 3 mM NADP.

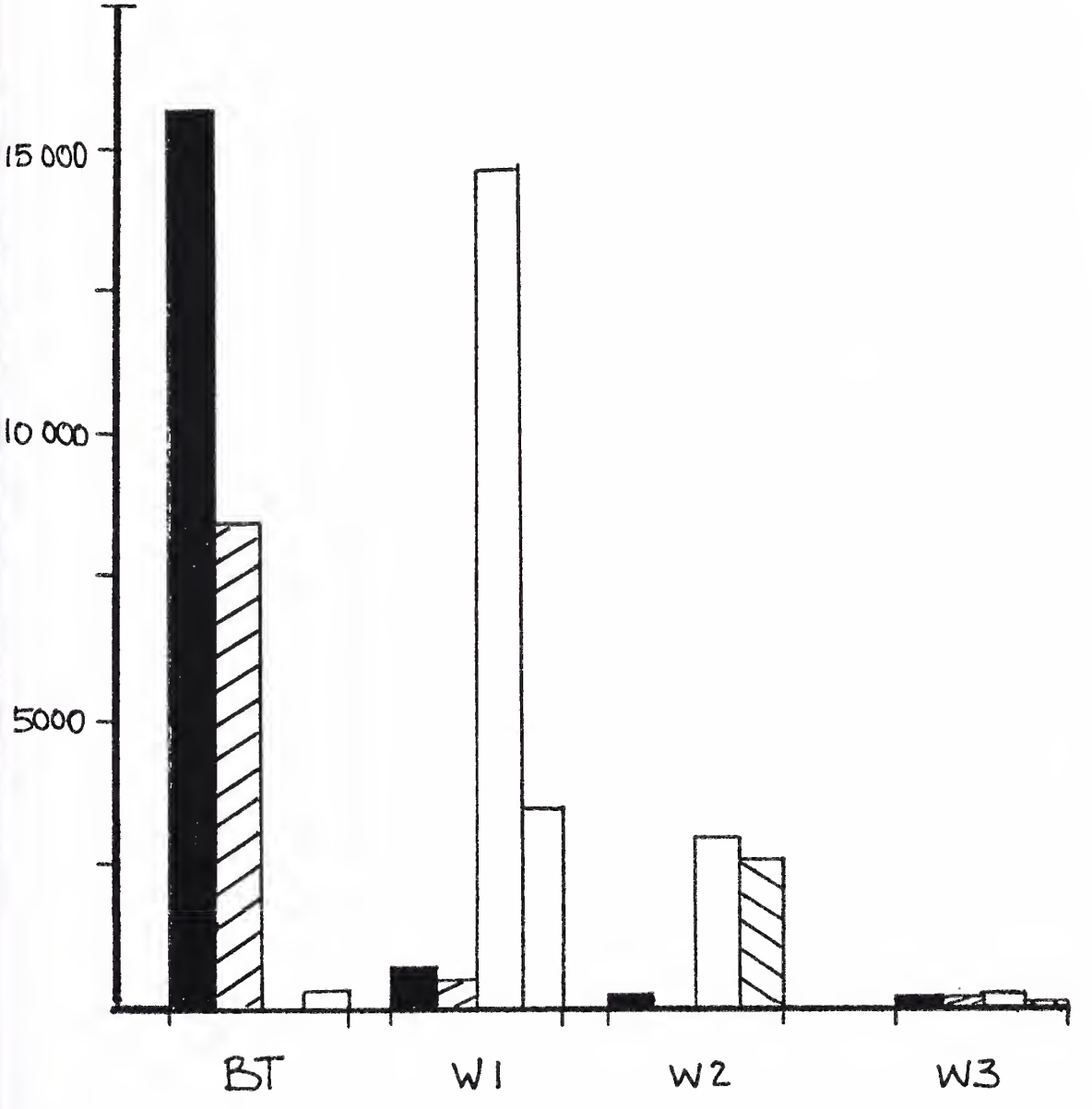


FIG. 11. DEAE-Sephadex column chromatography comparing the elution profile of deiodinase activity in the small column (with ▬ and without ▨ 3 mM NADP) versus the large column (5 X vols of small column) (with ▨ and without ▭ 3 mM NADP) by $^{125}I\text{-}T_4$ deiodinase assay in the (BT) "breakthrough" and (W1,W2,W3) "washes".

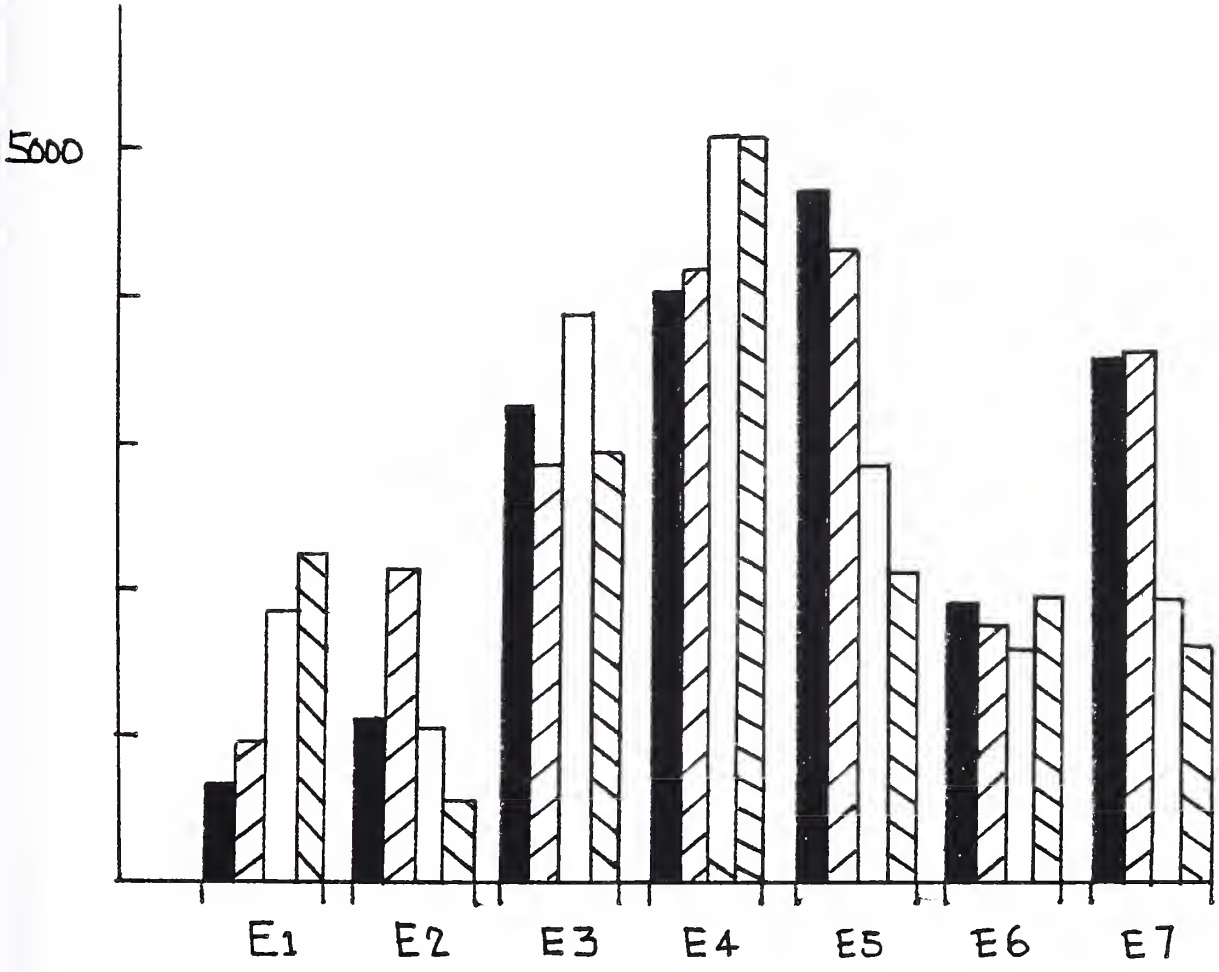


FIG. 12. DEAE-Sephadex column chromatography comparing the elution profile of deiodinase activity in the small column (with ▬ and without ▨ 3 mM NADP) versus the large column (with □ and without ▨ 3 mM NADP) by $^{125}I\text{-}T_4$ deiodinase assay in the (E1,E2,E3,E4,E5,E6,E7) eluates.

Table I. T_3 RIA Specificity :
Percent cross-reactivity with other iodothyronines

TYPE OF IODOTHYRONINE	PICOGRAMS OF IODOTHYRONINE[a]	PICOGRAMS OF TRIIODOTHYRONINE[b]	% CROSS-REACTIVITY[c]
T_2	1840	40.0	2.2
rT_3	2290	4.9	0.2
T_4	3110	33.0	1.1
T_3	120	118.6	98.8

[a,b] Picograms of indicated iodothyronine (a) detected as picograms of T_3 (b) by RIA.

[c] % Cross-reactivity = b/a X 100

Table II. T_3 RIA of microsomes, pH 4.5 supernatant and deiodinase A for T_3 production.

NADP/DTT[a]	MICROSOMES[b]	pH 4.5 SUPERNATANT[c]	DEIODINASE A[d]	CONTROL[e]
+	237	114.5	84.4	91.2
-	86.4	79.1	79.0	87.9

* Units in picograms T_3.
Incubation time = 20 min.

a In the presence (+) and absence (-) of 3 mM NADP and 5 mM DTT.

b,c,d Enzyme preparations, standard reaction mixes and assay conditions as per Methods.

e Control = 1 uM T_4 in standard reaction mixes without enzyme.

Table III. T_3 RIA of microsomes, pH 4.5 supernatant and dialyzed deiodinase A for T_3 production

NADP/DTT[a]	MICROSOMES[b]	pH 4.5 SUPERNATANT[c]	DIALYZED DEIODINASE A[d]	CONTROL[e]
+	227	140	112	86.0
-	89.0	81.0	85.0	73.0

* Units in picograms T_3.
Incubation time = 20 min.

a In the presence (+) and absence (-) of 3 mM NADP and 5 mM DTT.

b,c,d Enzyme preparations, standard reaction mixes and assay conditions as per Methods.

e Control = 1 uM T_4 in standard reaction mixes without enzyme.

Table IV. T_3 RIA of deiodinase A and B

NADP/DTT	DEIODINASE A	CONTROL
+	155.0	130.5
-	128.5	140.5

NADP/PL[a]	DEIODINASE B	CONTROL
+	124.0	124.8
-	120.5	122.0

* Units and symbols similar to previous tables.
Incubation time =1 hr.

[a] In the presence (+) and absence (-) of 3 mM NADP and 10 ul phospholipids mix as per Methods.

Table V. T_3 RIA of deiodinase A and B using 0.1 uM, 0.5 uM and 1.0 uM T_4 as substrate

NADP/DTT	0.1 uM T_4 DEIODINASE A	0.1 uM T_4 CONTROL	0.5 uM T_4 DEIODINASE A	0.5 uM T_4 CONTROL	1.0 uM T_4 DEIODINASE A	1.0 uM T_4 CONTROL
+	0-10	0-10	56.2	43.8	128	112
-	0-10	0-10	40.0	41.8	133	85.8

NADP/PL[a]	0.1 uM T_4 DEIODINASE A	0.1 uM T_4 CONTROL	0.5 uM T_4 DEIODINASE A	0.5 uM T_4 CONTROL	1.0 uM T_4 DEIODINASE A	1.0 uM T_4 CONTROL
+	44.3	0-10	50.0	37.8	118	112.9
-	0-10	0-10	52.0	47.8	116	-

* Units and symbols similar to previous tables.
Incubation time = 20 min.

[a] In the presence (+) and absence (-) of 3mM NADP and 10 ul phospholipids mix per Methods.

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